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SUMOylation of the Human Cytomegalovirus 72-Kilodalton IE1 Protein Facilitates Expression of the 86-Kilodalton IE2 Protein and Promotes Viral Replication

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The 72-kDa immediate-early 1 protein (IE1-72kDa) of human cytomegalovirus has been previously shown to be posttranslationally modified by covalent conjugation to the ubiquitin-related protein SUMO-1. Using an infectious bacterial artificial chromosome clone of human cytomegalovirus, we constructed a mutant virus (BAD*pm*IE1-K450R) that is deficient for SUMOylation of IE1-72kDa due to a single amino acid exchange in the SUMO-1 attachment site. Compared to wild-type virus, this mutant grew more slowly and generated a reduced yield in infected human fibroblasts, indicating that SUMO modification is required for the full activity of IE1-72kDa. The lack of SUMOylation did not affect the intranuclear localization of IE1-72kDa, including its ability to target to and disrupt PML bodies and to bind to mitotic chromatin. Likewise, SUMOylation-deficient IE1-72kDa activated several viral promoters as efficiently as the wild-type protein. However, the failure to modify IE1-72kDa resulted in substantially reduced levels of the IE2 transcript and its 86-kDa protein (IE2-86kDa). These observations suggest that SUMO modification of IE1-72kDa contributes to efficient HCMV replication by promoting the accumulation of IE2-86kDa.

Covalent attachment of ubiquitin (ubiquitylation) has long been known to serve as a tag for protein degradation via the 26S proteasome (reviewed in reference 59). Additionally, a number of ubiquitin-related proteins have been recently identified that are joined to other proteins through an enzymatic process that is biochemically analogous to, but functionally distinct from, ubiquitylation. Among the best characterized of these proteins are the small ubiquitin-like modifiers SUMO-1 (SMT3C), SUMO-2 (SMT3A), and SUMO-3 (SMT3B) (reviewed in references 29 and 60). While SUMOylation does not typically target proteins for degradation, it can have diverse effects on its substrates. SUMO modification enhances the stability of some cellular proteins (9, 16). In other cases, it modulates the subcellular localization and/or transactivation properties of its target proteins (reviewed in references 48, 67, and 71).

The list of substrates for SUMOylation includes an increasing number of viral regulatory proteins (reviewed in reference 72). Protein products of bovine papillomavirus E1 (51, 52), vaccinia virus E3L (56), adenovirus type 5 E1B (18), human herpesvirus 6 IE1 (21), and Epstein-Barr virus BZLF1 (1), as well as the major immediate-early proteins of human cytomegalovirus (HCMV), the 72-kDa immediate-early 1 protein (IE1-72kDa) and the 86-kDa immediate-early 2 protein (IE2-86kDa) (5, 26, 44, 63, 73), have all been shown to be targets of SUMO-1 conjugation. In addition, HCMV IE2-86kDa is modified by SUMO-2 and SUMO-3 (5, 26). While the functional consequences of SUMOylation are unknown for most of these viral proteins, transfection assays indicate that SUMO-1 modification is critical for nuclear import of the papillomavirus E1 protein (52) and for the nuclear accumulation, intranuclear targeting, and transforming functions of adenovirus E1B-55kDa (18). In contrast, SUMOylation does not obviously affect the subcellular localization of HCMV IE2-86kDa but enhances its transactivation capacity (5, 26). The biological consequences of SUMOylation for the life cycles of the respective viruses, however, remain to be determined, since in none of these cases has the SUMOylation site been mutated in the viral genome to allow for the study of this modification in the context of virus infection.

The UL123-coded IE1-72kDa and UL122-coded IE2-86kDa are abundant nuclear phosphoproteins expressed from alternatively spliced transcripts that originate from the major immediate-early locus of HCMV (reviewed in references 13 and 42). They share 85 amino-terminal amino acids corresponding to major immediate-early exons 2 and 3 but have distinct carboxy-terminal parts encoded by exon 4 (IE1) or exon 5 (IE2). Both proteins are believed to be important transcriptional regulators. IE2-86kDa interacts with multiple components of the cellular transcription machinery, promiscuously activating a wide range of viral and cellular promoters. Consistent with a role as the principal transcriptional activator of the HCMV lytic cycle, IE2-86kDa has been shown to be essential for early viral gene expression and productive viral growth in tissue culture (24, 38).

In transient-transfection assays IE1-72kDa can modestly augment transcription from a number of viral and cellular promoters, including the HCMV major immediate-early promoter, various HCMV early gene promoters, the SV40 promoter (reviewed in references 13 and 42), and the promoter of the human origin recognition complex 1 (hOrc-1) gene (62). Moreover, coexpression of IE1-72kDa can boost the transac-

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tivation capacity of IE2-86kDa. IE1-72kDa is not believed to bind DNA directly but physically interacts with several cellular transcription factors and other nuclear proteins, including CTF-1 (23), Sp-1 (76), E2F1 to -5 (39), $TAF_{II}130$ (37), p107 (49), Daxx (D. L. Woodhall, L. A. Teague, G. W. Wilkinson, S. Efstathiou, and J. H. Sinclair, presented at the 28th International Herpesvirus Workshop, Madison, Wis., 2003), and PML (2). The last two are key components of nuclear multiprotein complexes known by several names: PML bodies, PML oncogenic domains, or nuclear domain 10s (ND10s). ND10s have been implicated as sites for input viral genome deposition as well as for immediate-early transcription and initiation of viral DNA replication in HCMV and a number of other DNA viruses (reviewed in references 19 and 55). IE1-72kDa is known to be necessary and sufficient to disrupt ND10s during the early stages of HCMV infection and in transfected cells (4, 28, 31, 70). Intriguingly, IE1-72kDa can interact not only with the interchromatinic ND10 structures but also with chromatin, which results in colocalization with chromosomes during mitosis (2, 32, 70). The significance of these interactions with higher-order structures of the host cell nucleus is largely unknown, but it is tempting to speculate that they are linked to the transactivating properties of IE1-72kDa. Alternatively, they may be related to one or more of the other activities that have been ascribed to this viral protein. Specifically, IE1-72kDa has been shown to stimulate viral DNA replication (47, 58), affect cell cycle progression (14), block apoptosis (75, 79), cotransform cells to an oncogenic phenotype, and exhibit mutagenic activity (61). IE1-72kDa also displays kinase activity (46), and it is a dominant target for cell lysis by cytolytic $CD8^+$ T lymphocytes (reviewed in reference 53). The various activities that have been attributed to IE1-72kDa do not yet show a clear picture of this protein's role in the context of the viral life cycle. However, IE1-deficient mutant viruses display diminished replication efficiency, a decreased ability to form plaques, an inability to generate intranuclear replication compartments, and a broad defect in expression of viral early genes (20, 22, 43). This phenotype is especially pronounced when cells are infected at a low multiplicity of infection. Taken together, these observations suggest that IE1-72kDa is a pleiotropic regulator of the early events in the lytic infectious cycle whose functions are modulated by posttranslational modifications as well as noncovalent and covalent protein interactions.

Previous work has demonstrated that SUMO-1 conjugation occurs at lysine residue 450 (K450) within a SUMOylation consensus sequence in IE1-72kDa (63, 73). However, no functional consequences of this modification have been revealed (44, 63, 73). As is the case for all other known SUMOylated viral proteins, no recombinant virus with a specific mutation of the SUMOylation site in IE1-72kDa has been reported. Using an infectious bacterial artificial chromosome (BAC) clone of HCMV, we constructed a mutant (pBAD*pm*IE1-K450R) that is deficient for SUMOylation of IE1-72kDa due to a single amino acid exchange in the SUMO-1 attachment site, and we describe the mutation's phenotypic consequences.

MATERIALS AND METHODS

Cloning of IE1 expression plasmids. The plasmid pCGN-IE1 expresses HCMV IE1-72kDa fused to an amino-terminal influenza virus hemagglutinin (HA) epitope tag under control of the HCMV major immediate-early promoter-enhancer (79). The plasmid pCGN-IE1-K450R was derived from pCGN-IE1 by site-directed mutagenesis with the QuikChange procedure (Stratagene) according to the manufacturer's instructions. The oligonucleotides used for mutagenesis were K450R-fw (5′-GACACTGTGTCTGTCCGGTCTGAGCCAGTGTCTG-3′) and K450R-rv (5′-CAGACACTGGCTCAGACCGGACAGACACAGTGTC-3′). Error-free mutagenesis was verified by DNA sequence analysis of the entire IE1-72kDa-coding region.

For the construction of pEGFP-IE1, which expresses wild-type IE1-72kDa fused to the carboxy terminus of the enhanced green fluorescent protein (EGFP), the IE1-coding sequence was released from pCGN-IE1 with KpnI and BamHI and inserted into the same sites of pcDNA3 (Invitrogen). From the resulting plasmid, pcDNA-IE1, the insert was subsequently excised with HindIII and BamHI and inserted in frame into the HindIII and BamHI sites of pEGFP-C1 (Clontech). To generate plasmid pEGFP-IE1-K450R, which expresses the respective IE1-72kDa mutant fused to EGFP, pEGFP-C1 was cut with SacI and BamHI and ligated with the SacI-BamHI fragment from pCGN-IE1-K450R.

Mutagenesis of HCMV genomes. HCMV mutants were produced by using a BAC clone of the HCMV AD169 genome (AD169-BAC [25], referred to as pBAD*wt* in this publication). They were generated by homologous recombination in *Escherichia coli* with linear, PCR-generated DNA fragments (74, 77; reviewed in references 12, 40, and 68).

For the generation of the IE1 knockout deletion mutant BAD*sub*IE1, an ampicillin resistance (*amp*) gene was excised from vector pST76A (50) by using the restriction enzymes EcoRI and NotI and inserted into the MfeI and NotI sites of vector pECFP-N1 (Clontech), downstream of the enhanced cyan fluorescent protein (ECFP) gene. The resulting plasmid, pECFP-*amp*, served as a template for PCR amplification of the ECFP-coding sequence linked to the *amp* cassette by using purified primers that contained 50 nucleotides for homologous recombination with the DNA sequences flanking IE1 exon 4 in addition to 20 or 21 nucleotides for hybridization with the PCR template (IE1-ECFP-*amp*-fw, 5′-AGGAGGACGGATACTTATATGTGTTGTTATCCTCCTCTACAGTCAAACAGAATTCGGTGAGCAAGGGCGAGGAGCTG-3′; IE1-ECFP-*amp*-rv, 5′-GTGACGTGGGATCCATAACAGTAACTGATATATATATACAATAGTTTAGCAAGTGGCACTTTTCGGGG-3′). Additionally, an EcoRI site was introduced into primer IE1-ECFP-*amp*-fw to facilitate identification of recombinant BACs by restriction digestion. Allelic exchange of BACs with linear DNA fragments was carried out in *E. coli* strain DY380, which expresses the recombination genes *exo*, *bet*, and *gam* of bacteriophage λ in a temperature-dependent fashion, as described in detail elsewhere (34, 74). BAC DNAs from a number of ampicillin-resistant colonies were prepared, digested with different restriction enzymes, and separated on 0.6% agarose gels to verify the success of the recombination procedure and the overall integrity of the viral genome. The accuracy of mutagenesis was further confirmed by PCR and DNA sequence analyses.

For the construction of the IE1 point mutant BAC pBAD*pm*IE1-K450R and the revertant pBAD*rev*IE1, a BamHI site was introduced into plasmid pCGN-IE1 directly after the stop codon of the IE1-coding sequence by using the QuikChange mutagenesis strategy (primers were IE1-B-fw [5′-GCAAGGCTGACCAGTAAGGATCCGTATATATATATCAG-3′] and IE1-B-rv [5′-CTGATATATATATACGGATCCTTACTGGTCAGCCTTGC-3′]) to generate pCGN-IE1-B. Likewise, one EcoRI site was converted into a BamHI site in plasmid pSLFRTKn (6) by using primers FKF-B-fw (5′-CGTCGTGGAATGCCTTCGGATCCGAAGTTCCTATACTTTC-3′) and FKF-B-rv (5′-GAAAGTATAGGAACTTCGGATCCGAAGGCATTCCACGACG-3′). This was followed by excision of the kanamycin resistance (*kan*) gene flanked by FLP recognition target (FRT) sites from this plasmid with BamHI and insertion into the BamHI site of pCGN-IE1-B. The resulting plasmid (pCGN-IE1-B-*kan*) was used as a template for QuikChange mutagenesis with primers K450R-fw and K450R-rv. This procedure resulted in plasmid pCGN-IE1-K450R-B-*kan*, containing two nucleotide substitutions in the IE1-coding sequence, i.e., AAG to CGG in codon 450. All mutations were verified by comprehensive DNA sequence analysis. Fragments comprising the mutated IE1 sequences linked to the FRT-flanked *kan* gene were PCR amplified by using templates pCGN-IE1-B-*kan* and pCGN-IE1-K450R-B-*kan* together with primers IE1-Kan-fw (5′-AGGAGGACEGATACTTATATGTGTTGTTATCCTCCTCTACAGTCAAACAGATTAAGGTTCGAGTGG-3′) and IE1-Kan-rv (5′-GTGACGTGGGATCCATAACAGTAACTGATATATATATATACAATAGTTTAAGGACGACGACGACAAGTAA-3′). Again, these oligonucleotides contained an additional 50 nucleotides for homologous recombination. Linear recombination with the purified PCR products and pBAD*sub*IE1 in *E. coli* DY380 was performed as described above, and the integrity of BAC DNAs (pBAD*pm*IE1-K450R-*kan* and pBAD*rev*IE1-*kan*) from ampicillin-sensitive, kanamycin-resistant bacterial colonies was confirmed by restriction digestion. DNAs from selected BAC clones were subsequently transformed into *E. coli* strain DH10B, and

kan cassettes were removed by FRT site-directed recombination with FLP recombinase expressed from plasmid pCP20 (15). This plasmid replicates in a temperature-dependent fashion, allowing FLP-mediated recombination at 30°C (permissive temperature) and its own elimination at 42°C (restrictive temperature). Single colonies were screened for kanamycin sensitivity, and BACs from selected colonies were further analyzed by restriction digestion and DNA sequence analyses.

Cell culture and virus infections. The following cell types were used in this study: primary human foreskin fibroblasts (passages 5 to 18), ihf-2 cells (a fibroblast cell line that was immortalized by introduction of the human papillomavirus E6 and E7 genes) (22), ihfie1.3 cells (a gift from E. Mocarski, Stanford University), E6/E7-immortalized fibroblasts that stably express HCMV IE1-72kDa (43), and the human non-small-cell lung carcinoma cell line H1299 (41). All cells were cultured as monolayers in medium containing 10% fetal calf serum at 37°C.

HCMV was grown and titers were determined on fibroblasts by standard procedures. For viral growth analyses, infected cells and culture medium were combined and subjected to one freeze-thaw cycle followed by a short sonication step, and virus titers were determined according to the median tissue culture infectious dose method (54) on fibroblasts (BAD*wt* and BAD*rev*IE1) or ihfie1.3 cells (BAD*pm*IE1-K450R). To reconstitute infectious virus from wild-type and mutant viral genomes, BAC DNA was purified by using the NucleoBond plasmid kit (BD Biosciences Clontech), and 2 μg of this DNA was transfected into fibroblasts by electroporation as previously described (7). Along with the BAC DNA, 1 μg of pCGN-pp71, which enhances the infectivity of HCMV DNA (7), and 1 μg of the *cre*-expressing plasmid pBRep-Cre (25), to direct excision of the BAC sequences from the viral genome, were included in the transfection mix.

Fluorescence microscopy. For indirect immunofluorescence analyses, subconfluent fibroblasts received 5 μg of plasmid DNA by electroporation as previously described (7). Following transfection, cells were plated on coverslips. Alternatively, cells were infected with BAD*pm*IE1-K450R-1 or BAD*wt* at a multiplicity of 3 or 0.01 PFU/cell. At different times postinfection, cells were washed twice with phosphate-buffered saline (PBS) and fixed with paraformaldehyde (2% in PBS) for 15 min at room temperature, followed by three 5-min washes with PBS and permeabilization with 0.1% Triton X-100 in PBS for 15 min. After three washes in PBS the permeabilized cells were blocked for 1 h in PBS containing 2% bovine serum albumin and 0.05% Tween 20. After that, samples were washed once in PBS and reacted for 1 h at room temperature with the appropriate primary antibodies in a humidity chamber, followed by three 5-min washes with PBS and a 1-h incubation with the appropriate Alexa 488- or Alexa 546-conjugated secondary antibody (Molecular Probes). After three additional washes in PBS, coverslips were mounted in slow-fade solution (Molecular Probes), and images were acquired on a Nikon TE200 fluorescence microscope with a charge-coupled device camera (Diagnostic Instruments) or a Zeiss LSM laser scanning microscope. The primary antibodies used in this study were rat anti-HA purified monoclonal antibody (3F10; Roche), mouse anti-IE1 monoclonal hybridoma supernatant (1B12) (79), mouse anti-PML monoclonal hybridoma supernatant (5E10) (65), and rabbit anti-Sp100 polyclonal serum (AB1380; Chemicon).

For live-cell visualization of EGFP fusion proteins, H1299 cells were grown on glass-bottom dishes (MatTek), and approximately 50% confluent cultures were transfected with 5 μg of plasmid pEGFP-IE1 or pEGFP-IE1-K450R by using a modified calcium phosphate precipitation procedure. At about 48 h after transfection, cells were stained with Hoechst 33342 according to the manufacturer's instructions, and confocal images were acquired on a Zeiss LSM 510 laser scanning microscope. ECFP expressed from recombinant virus BAD*sub*IE1-1 was detected 3 days after transfection of the respective BAC DNA (2 μg) into fibroblasts by electroporation (7).

Reporter gene assays. For luciferase assays, subconfluent fibroblast monolayers were transfected by using the Fugene 6 reagent (Roche) with 1 μg of reporter and 0.1 μg of effector plasmid DNA according to the manufacturer's instructions. Plasmid pCGN-IE2 expresses HCMV IE2-86kDa under control of the HCMV major immediate-early promoter-enhancer and has been previously described (79). The following reporter plasmids were used in this study: pGL3-Promoter (Promega), pGL3-ICP36 (57), pGL3-MIEP (57), and pHsOrc1Luc (62). Infections (multiplicity of infection [MOI] = 3 PFU/cell) were performed 24 h after transfection of reporter plasmids, and cells were collected at 18 h postinfection.

Western blot analyses. To assay the accumulation of viral proteins in HCMV-infected cells, lysates were prepared at various times after infection by suspending equal amounts of cells directly in 2× sample buffer (100 mM Tris [pH 6.8], 200 mM dithiothreitol, 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol, 40 μM *N*-ethylmaleimide), followed by heating of the samples at 95°C for 10 min, quick sonication, and additional heating at 95°C for 10 min. Aliquots were separated in sodium dodecyl sulfate-containing 10% polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes. Nonspecific binding was blocked by incubation in PBS containing 5% nonfat dry milk for at least 2 h. After that, membranes were washed once in PBS and probed with primary mouse monoclonal antibodies specific for the following viral or cellular proteins for 1 to 12 h at room temperature or 4°C, respectively: α-tubulin (DM1A; Sigma), IE1 (1B12) (79), IE1/IE2 (MAB810; Covance), SUMO-1 (21C7; Zymed), and ppUL44 (anti-ICP36; Virusys). After three washes with PBS containing 0.1% Tween 20, membranes were incubated with anti-mouse immunoglobulins conjugated with horseradish peroxidase (Dako) for 1 h, followed by three washes with PBS–0.1% Tween 20 and chemiluminescence detection (Super Signal; Pierce).

Northern blot hybridization. Cells on 15-cm-diameter dishes were scraped into 10 ml of Trizol reagent (Gibco Life Sciences) at the indicated times after infection and stored at −80°C. After completion of the time course, samples were thawed, and RNA was purified by using the Trizol protocol. Then, 10 μg of each purified RNA preparation was electrophoretically separated on formaldehyde gels and blotted to Hybond N+ membranes (Amersham). DNA probes were generated and nonradioactively labeled with digoxigenin-11-dUTP (Roche) by PCR with the indicated cDNAs (IE1 exon 4, IE2 exon 5, UL37 exon 1, TRS1, and UL44) in pGEM-T (Promega) (11) as templates and universal primers for the T7 and Sp6 promoter sequences. Membranes were hybridized to the labeled probes overnight at 42°C in DIG Easy Hyb buffer (Roche). Washing and antibody detection were performed as recommended by the manufacturer.

RESULTS

Construction of HCMV BACs with mutations in the IE1 gene. It has been previously demonstrated that posttranslational modification of IE1-72kDa by conjugation to SUMO-1 occurs at a single acceptor site (K450), and mutation of K450 to arginine (K450R) results in loss of IE1-72kDa SUMOylation in transfected cells and in cell-free assays (63, 73). Based on these observations, we employed the BAC system to introduce two nucleotide exchanges into the viral genome that result in the expression of a K450R mutant IE1-72kDa. A three-step strategy was employed, which allows for the introduction of any point mutation into the viral BAC without requiring the presence of restriction enzyme sites, further cloning, or shuttle plasmids. Instead, the first two recombination steps were performed by allelic exchange with linear, PCR-generated fragments, and the third step involved site-specific excision by the FLP recombinase. To our knowledge, this three-step method has not been applied before to the mutagenesis of viral genomes.

In step one, we generated three mutant BAC clones (pBAD*sub*IE1-1 to -3) in which almost all of the IE1-specific exon 4 sequence (except the nucleotides encoding the first three amino acids and the stop codon) was replaced by an *amp* gene and an ECFP-coding sequence (Fig. 1A). The ECFP sequence was introduced to facilitate the identification of infected cells by fluorescence microscopy and was inserted in a way that permits splicing to major immediate-early exon 3 and in-frame translation as a fusion protein comprising an 85-residue amino-terminal peptide (expressed from major immediate-early exons 2 and 3) and the fluorescent protein. Accordingly, after transfection of pBAD*sub*IE1 DNAs into fibroblasts, transfected cells displayed predominantly nuclear fluorescence (data not shown), consistent with the earlier proposal that amino acids 1 to 24 of IE1-72kDa comprise a nuclear localization signal (70). The identity and integrity of the mutant BACs were further confirmed by restriction digestion with different enzymes (Fig. 1B and data not shown) and PCR analysis (Fig. 1C).

In step two, linear recombination was used to replace the

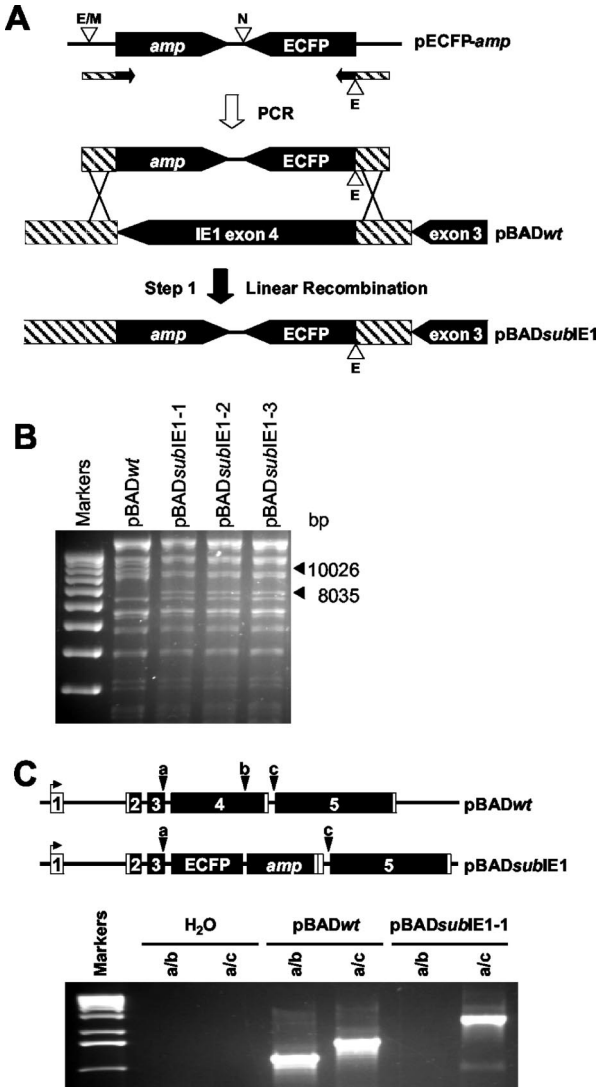

FIG. 1. Generation and characterization of pBAD*sub*IE1 mutant BACs. (A) Introduction of a targeted substitution mutation. The positions of restriction enzyme sites used for cloning are indicated by capital letters (E, EcoRI; M, MfeI; N, NotI). Black block arrows, ECFP, *amp*, and major immediate-early exon 4 or exon 3 DNA sequences, with the arrowheads pointing in the sense-strand directions; hatched segments, introns that flank the IE1-specific exon 4, where the crossover events (indicated by crossed lines) during homologous recombination in *E. coli* occurred; narrow arrows, PCR primers. Elements in this diagram are not drawn to scale. (B) Restriction digest with EcoRI of DNA from the wild-type HCMV BAC and three independent mutant BAC clones. In all three mutants, due to an EcoRI site that was recombined with the ECFP-*amp* cassette, the 10,026-bp band (arrowhead) present in the wild-type disappears. Instead, two bands of 8,035 bp (arrowhead) and 3,306 bp (not visible) appear. Markers are a 1-kb ladder. (C) PCR analysis with the wild-type BAC and one of the mutant BAC clones or without template (H_2O). PCR primers are designated a, b, and c (a, IEP3C; b, IEP4J; c, IEP5B) (30), and their approximate positions within the HCMV major immediate-early region are indicated by arrowheads. Boxes represent exons of the HCMV major immediate-early region (the numbers 1 to 5 correspond to major immediate-early exons 1 to 5) or the ECFP and *amp* genes (coding sequences are shown in black, and noncoding sequences are shown in white). Lines represent introns or other noncoding sequences. An ethidium bromide-stained 1% agarose gel shows PCR products of the expected sizes amplified with the indicated primer combinations and templates (lane 4, 1,158 bp; lane 5, 1,786 bp; lane 7, 3,105 bp). As predicted, the reactions in lanes 2, 3, and 6 did not result in amplification products. Markers (lane 1) are a 1-kb ladder.

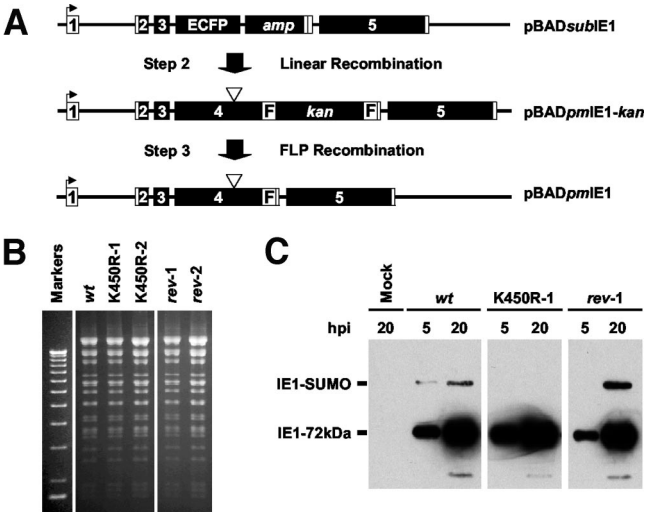

FIG. 2. Construction and analysis of IE1 point mutant BACs and viruses. (A) Diagram showing the recombination strategy that was used to generate pBAD*pm*IE1-K450R from pBAD*sub*IE1. Boxes with numbers represent major immediate-early exons 1 to 5. The ECFP, *amp*, and *kan* genes are also shown as boxes. Open triangles symbolize specific point mutations, and FRT sites are represented by small white boxes with the letter F inside (coding sequences are shown in black, and noncoding sequences are shown in white). Lines represent introns or other noncoding sequences. (B) Restriction digests of wild-type (*wt*, pBAD*wt*) and mutant (K450R, pBAD*pm*IE1-K450R; *rev*, pBAD*rev*IE1) BAC DNAs (two independently generated clones per mutant are designated with the suffix -1 or -2) with EcoRI followed by electrophoresis in a 0.6% ethidium bromide-stained agarose gel. All BAC preparations show a wild-type pattern of restriction fragments. Markers are a 1-kb ladder. (C) Western blot analysis with anti-IE1 antibody 1B12 and whole-cell lysates from uninfected cells (Mock) or fibroblasts infected with BAD*wt* (*wt*), BAD*pm*IE1-K450R-1 (K450R-1), and BAD*rev*IE1-1 (*rev*-1) at an MOI of 3 PFU/cell. The blots show bands corresponding to the SUMOylated and/or nonconjugated IE1-72kDa at 5 or 20 h postinfection (hpi).

ECFP-*amp* cassette in pBAD*sub*IE1-1 with an IE1 exon 4 sequence that was modified to contain the desired nucleotide substitutions corresponding to the K450R amino acid change (Fig. 2A). Attached to the mutated IE1 gene, a *kan* cassette that was flanked by FRT sites was inserted, resulting in pBAD*pm*IE1-K450R-*kan*. In step 3, the FRT sites allowed for subsequent excision of the *kan* gene by expression of the site-specific FLP recombinase, thus leaving only viral sequences at the mutation site and a small insertion of 54 nucleotides corresponding to a single FRT site (34 bp) plus the primer binding site (20 bp) used for generating the PCR fragments (Fig. 2A). Following this three-step procedure, two independent clones of pBAD*pm*K450R were generated. To control for potential functional effects of the 54 nucleotide insertion, two revertant BACs (pBAD*rev*IE1) were constructed from pBAD*sub*IE1-1 by using the wild-type IE1 sequence for allelic exchange. Again, the identity and integrity of all recombinant BACs were verified by various restriction digestions and DNA sequence analysis (Fig. 2B and data not shown).

Infectivity of mutant BACs. We electroporated purified pBAD*wt*, pBAD*sub*IE1, pBAD*pm*IE1-K450R, and pBAD*rev*IE1 DNAs into permissive fibroblasts and monitored the transfected cultures for plaque development due to reconstituted

TABLE 1. Virus titers at 20 days after transfection of fibroblasts with HCMV BAC constructs[a]

BAC construct	Virus titer (median tissue culture infective dose) in expt:			
	1	2	3	4
pBAD*wt*	5.4×10^6	6.8×10^6	3.2×10^6	5.3×10^6
pBAD*sub*IE1-1	0	ND[b]	0	0
pBAD*sub*IE1-2	0	0	ND	ND
pBAD*sub*IE1-3	0	ND	0	ND
pBAD*pm*IE1-K450R-1	5.0×10^5	5.2×10^5	4.4×10^5	2.8×10^5
pBAD*pm*IE1-K450R-2	3.1×10^5	1.0×10^5	ND	ND
pBAD*rev*IE1-1	4.7×10^6	4.2×10^6	6.1×10^6	3.0×10^6
pBAD*rev*IE1-2	7.9×10^6	2.2×10^6	ND	ND

[a] Multiple independently generated mutant clones were tested.
[b] ND, not determined.

virus. The transfection experiments were repeated multiple times with several independently generated BAC clones and preparations to minimize the chance that negative results were due to deficiencies in the integrity or quality of BAC DNAs. After 7 to 10 days, nascent plaques could be identified, and at 14 days after transfection, infectious virus was quantified from the supernatant of the cultures. In the four representative experiments shown in Table 1, and in several additional transfections, the pBAD*wt*, pBAD*pm*IE1-K450R, and pBAD*rev*IE1 clones consistently yielded infectious virus, although the titers of the reconstituted wild-type and revertant viruses were about 10-fold higher than those of the K450R mutants. In contrast, pBAD*sub*IE1 never generated detectable virus. The failure to recover infectious virus from these transfections argues that a functional IE1 gene is essential for the full infectivity of HCMV BAC DNA. Moreover, these experiments show that SUMO modification is not absolutely required for the function of IE1-72kDa during lytic infection.

K450R mutant viruses display attenuated growth. After virus reconstitution from pBAD*wt*, pBAD*pm*IE1-K450R, and pBAD*rev*IE1, virus stocks for which the titers were determined were prepared, and the lack of IE1-72kDa SUMOylation in the K450R mutant virus was confirmed by Western blotting with an IE1-specific monoclonal antibody (Fig. 2C). Even after overexposure of the protein blot, no ~92-kDa high-molecular-mass band, which has been shown previously to correspond to SUMOylated IE1-72kDa (44, 63, 73), was present in the K450R mutant under conditions where this species was easily detected in cells infected with the wild-type and revertant viruses (Fig. 2C). Likewise, an antibody directed against SUMO-1 could not detect any modified IE1 protein in cells infected with BAD*pm*IE1-K450R-1 (data not shown).

Subsequently, single-step (multiplicity of 3 PFU/cell) and multistep (multiplicity of 0.01 PFU/cell) growth analyses were performed. The single-step analyses were done both with complementing, immortalized fibroblasts that stably express wild-type IE1-72kDa (ihfie1.3 cells) (43) and with noncomplementing parental cells (ihf-2 cells) (22). For the multistep growth curves, primary fibroblasts were used. Compared to the wild-type and revertant viruses, the IE1-K450R mutant displayed slower replication at both low and high MOIs, with an about 10-fold difference in virus titers on noncomplementing cells (Fig. 3A and B). These observations are consistent with the

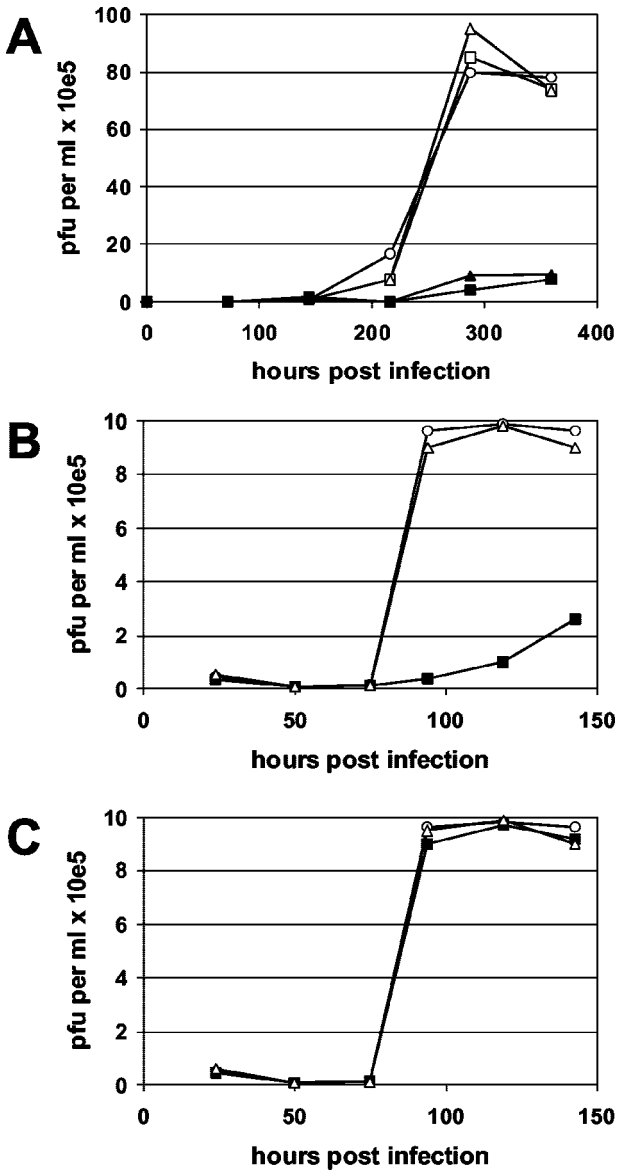

FIG. 3. Growth characteristics of SUMOylation-deficient IE1 mutant HCMVs compared to wild-type and revertant viruses. Symbols represent average values from two experiments performed in parallel. Open circles, BAD*wt*; open triangles, BAD*rev*IE1-1; open squares, BAD*rev*IE1-2; filled squares, BAD*pm*IE1-K450R-1; filled triangles, BAD*pm*IE1-K450R-2. (A) Growth kinetics after low-multiplicity infection (0.01 PFU/cell) of fibroblasts. (B) Growth kinetics after high-multiplicity infection (3 PFU/cell) of ihf-2 cells. (C) Growth kinetics after high-multiplicity infection (3 PFU/cell) of complementing ihfie1.3 cells.

results obtained by quantitation of reconstituted virus after BAC transfection (Table 1). Importantly, the K450R-specific growth defect was almost completely offset in cells exogenously expressing IE1-72kDa (Fig. 3C). Moreover, in the multistep experiment, similar growth defects could be detected with mutant viruses derived from two independent pBAD*pm*IE1-K450R clones, while two different revertant viruses (BAD*rev*IE1) showed growth kinetics that were very similar to those of the wild type (Fig. 3A). These results indicate that the observed

phenotype of the K450R mutant viruses is a specific effect due to the point mutation in IE1-72kDa, demonstrating that SUMOylation of this viral protein promotes efficient virus replication in fibroblasts.

Subcellular localizations and nuclear interactions of the IE1-72kDa mutant proteins. Previous work has shown that modification by SUMO-1 is critical for the (sub)nuclear targeting of some viral proteins (18, 52), and SUMOylation of the cellular PML protein is known to be important for its association with ND10s (17, 33, 45, 78). IE1-72kDa is transiently colocalized with PML at ND10s in the first hours after infection, and subsequently the two proteins are redistributed, displaying a uniform nuclear diffuse pattern (4, 28, 31, 70). Thus, to investigate how SUMO conjugation contributes to the function of IE1-72kDa, we asked whether the SUMOylation-deficient K450R mutant protein can still target to the nucleus and disrupt ND10s as efficiently as wild-type IE1-72kDa. For this purpose, we transfected fibroblasts with plasmids expressing wild-type IE1-72kDa and IE1-K450R and performed double-label immunofluorescence analyses with antibodies against the IE1-72kDa and PML proteins. We found that IE1-K450R was still able to efficiently target to the nucleus, localizing in a diffuse pattern that was indistinguishable from the wild-type pattern, indicating that the mutant protein retained the ability to trigger the redistribution of PML (Fig. 4A). These observations confirm results of previous studies that have been carried out with transfected cells (44, 63, 73).

To verify these observations in the context of an HCMV infection, we exposed fibroblasts to equal amounts of the wild-type and K450R mutant viruses (MOI = 3 PFU/cell) and monitored the temporal subnuclear distribution patterns of IE1-72kDa and Sp100, an alternative marker for ND10s (66), over a time course of 24 h. As expected, at 1 h after infection Sp100 was found predominantly in the typical nuclear dot-like staining pattern corresponding to ND10s, and there was no detectable expression of wild-type or mutant IE1-72kDa (Fig. 5a to d and m to p). At 6 h postinfection, both the wild-type and the IE1-K450R proteins accumulated in the host cell nucleus. Both proteins were found to colocalize with Sp100 in either nuclear diffuse or punctate patterns, indicating that at this stage ND10 disruption was complete in some but not all of the infected cells (Fig. 5e to h and q to t). Finally, at 24 h postinfection, ND10 disruption was equally complete in all cells treated with the BAD*wt* or the BAD*pm*IE1-K450R virus, as the Sp100 protein was displaced from ND10s together with wild-type IE1-72kDa and IE1-K450R in all infected nuclei, although some less homogenous Sp100 staining remained (Fig. 5i to l and u to x). Similar results were obtained with infections performed at a low MOI of 0.01 PFU/cell (data not shown). These observations clearly show that lack of SUMOylation has no detectable effect on ND10 disruption by IE1-72kDa.

Besides its interaction with interchromatinic ND10s, IE1-72kDa is also known to interact with cellular chromatin, at least during mitosis (2, 32, 70). Since infected cells do not usually undergo mitosis (10, 35, 36, 69), we analyzed chromatin association of wild-type and K450R mutant IE1-72kDa by comparing the localizations of these proteins fused to EGFP in living transfected cells. As shown in Fig. 4B, both the wild-type (panel a) and the mutant (panel b) fusion proteins colocalized very efficiently with condensed chromatin in transfected cells undergoing mitosis. Taken together, these results show that lack of SUMOylation has no obvious effects on the subnuclear distribution of IE1-72kDa, including its ability to target to and disrupt ND10s and to associate with mitotic chromatin. Therefore, it is unlikely that the observed growth defect of BAD*pm*IE1-K450R is due to an altered localization of the mutant IE1-72kDa.

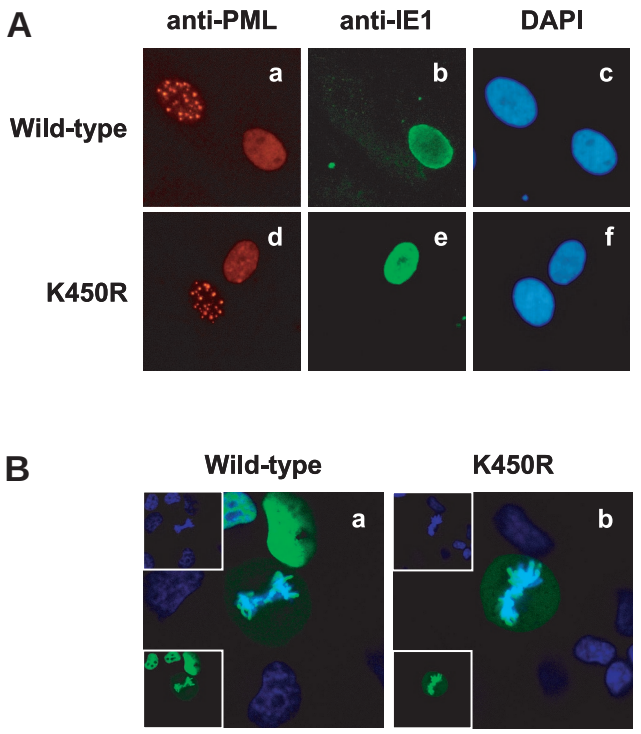

FIG. 4. Subcellular localizations and intranuclear interactions of wild-type and mutant IE1-72kDa proteins in transfected cells. (A) Fibroblasts were electroporated with plasmid pCGN-IE1 (panels a to c) or pCGN-IE1-K450R (panels d to f), and immunofluorescent double-labeling was performed with mouse anti-PML (5E10) (panels a and d) and rat anti-HA (3F10) (panels b and e) antibodies, followed by incubation with anti-mouse Alexa 546 (panels a and d) or anti-rat Alexa 488 (panels b and e) conjugates and DNA staining with DAPI (4′,6′-diamidino-2-phenylindole) (panels c and f). Magnification, ×200. (B) H1299 cells were transfected with plasmids pEGFP-IE1 (panel a) and pEGFP-IE1-K450R (panel b), and DNA was stained with Hoechst 33342. Living cells that were both successfully transfected and undergoing mitosis were observed by live-cell confocal microscopy. Merged images are shown, with single-color pictures presented as smaller insets (EGFP, lower left corner; Hoechst 33342, upper left corner).

Transactivating properties of the mutant proteins. IE1-72kDa can activate transcription from a variety of HCMV and other promoters in transient-transfection assays, on its own or in concert with IE2-86kDa (reviewed in references 13 and 42). IE1-72kDa is also required for efficient viral early gene expression in infected cells (20, 22). To investigate whether the K450R mutation affects the transactivation capacity of IE1-72kDa, we performed luciferase reporter assays in transiently transfected cells by using various viral and cellular promoter constructs. As expected, the wild-type IE1-72kDa protein was able to activate the SV40 early promoter, the HCMV UL44 promoter, the HCMV major immediate-early promoter, and the cellular hOrc-1 promoter by factors of 2.6 to 16.1, depending on the reporter construct (Fig. 6A). IE1-K450R displayed

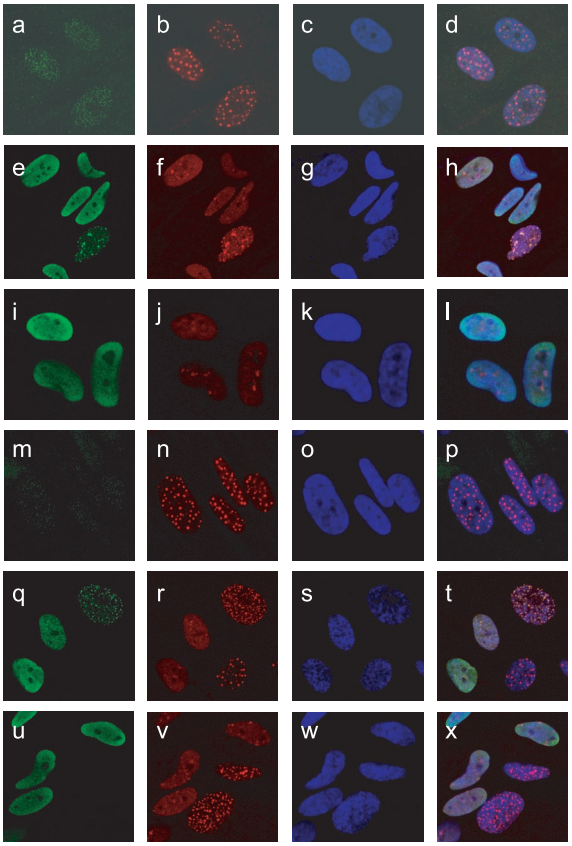

FIG. 5. ND10 interaction of wild-type and mutant IE1-72kDa proteins in HCMV-infected cells. Fibroblasts were infected with BAD*rev*IE1-1 (a to l) or BAD*pm*IE1-K450R-1 (m to x) at a multiplicity of 3 PFU/cell and fixed 1 h (a to d and m to p), 6 h (e to h and q to t), or 24 h (i to l and u to x) later. Accumulation of IE1-72kDa proteins and disruption of ND10s were monitored with antibody combinations mouse anti-IE1 (1B12)–anti-mouse Alexa 488 (a, e, i, m, q, and u) and rabbit anti-Sp100–anti-rabbit Alexa 546 (b, f, j, n, r, and v), respectively. DNA was simultaneously stained with DAPI (c, g, k, o, s, and w). Merged images are also shown (d, h, l, p, t, and x).

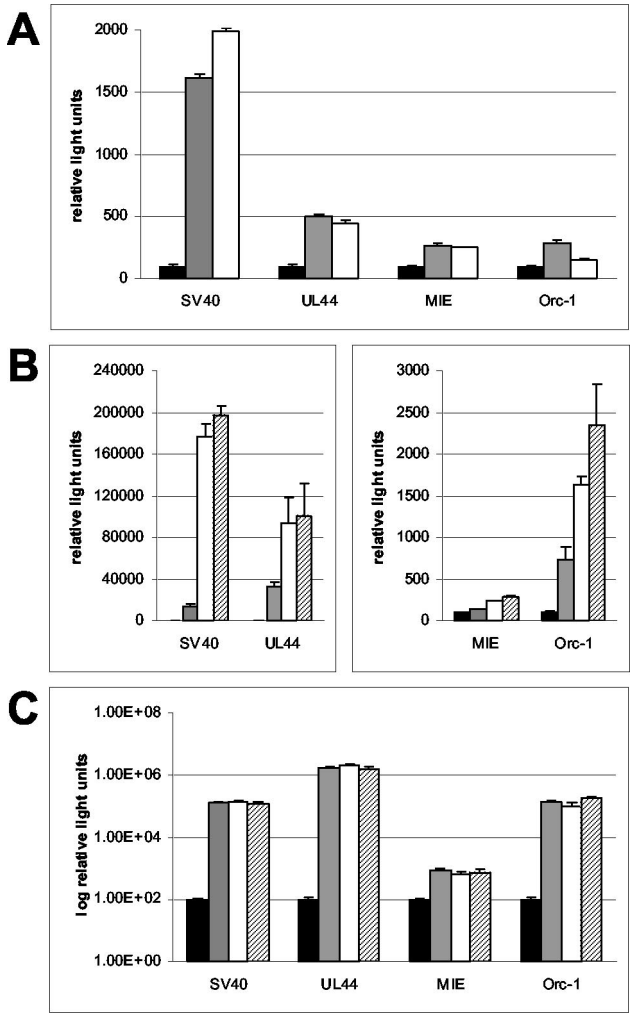

FIG. 6. Transcriptional activation by wild-type and mutant IE1-72kDa proteins. Fibroblasts were transfected with luciferase reporter plasmid pGL3-Promoter (SV40), pGL3-ICP36 (UL44), pGL3-MIEP (MIE), or pHsOrc1Luc (Orc-1). Bars represent average values and standard errors from three separate transfections. Relative light units are presented as percentages of the respective control value, which was set to 100%. (A) Respective reporter plasmids were contransfected with pCGN (black bars) (set to 100%), pCGN-IE1 (gray bars), or pCGN-IE1-K450R (white bars). (B) Reporter plasmids were cotransfected with pCGN (black bars) (set to 100%) (hardly visible in left panel), pCGN-IE2 (gray bars), pCGN-IE2 and pCGN-IE1 (white bars), or pCGN-IE2 and pCGN-IE1-K450R (hatched bars). (C) Transfections with reporter plasmids were followed by infections with BAD*wt* (gray bars), BAD*pm*IE1-K450R-1 (white bars), or BAD*rev*IE1-1 (hatched bars) or mock infection (black bars) (set to 100%).

wild-type-like activity on all three viral promoters, although transactivation was modestly reduced at the cellular hOrc-1 promoter (1.6- versus 2.8-fold activation) (Fig. 6A). Similar results were obtained when the same promoters were tested for synergistic activation by wild-type or mutant IE1-72kDa and IE2-86kDa, although the SUMOylation-deficient protein had a tendency to be a slightly more efficient activator in these assays (Fig. 6B). To confirm these results in the context of viral replication, we transfected cells with reporter plasmids and then infected them with the wild-type, K450R, and revertant viruses. As expected, there were no significant differences in promoter activation by the three viruses (Fig. 6C). These results suggest that SUMOylation is not required for transactivation of viral and cellular promoters by IE1-72kDa.

Reduced expression of IE2-86kDa in the IE1-K450R mutant virus. The fact that we did not find significant differences between the transactivating properties of the wild-type and the K450R mutant IE1-72kDa in our reporter assays does not rule out possible effects on viral gene expression due to differential regulation of promoters that have not been tested or posttranscriptional events. Therefore, we compared the levels of several viral transcripts by Northern blotting at various times after infection of fibroblasts with BAD*pm*K450R-1 or BAD*rev*IE1-1. The expression kinetics of two immediate-early transcripts, UL37 and TRS1 (Fig. 7A), as well as the early UL44 mRNA (not shown), were indistinguishable between the mutant and revertant viruses. However, the amounts of IE2 mRNA were substantially reduced in the K450R mutant at all times assayed. The levels of IE1 mRNA were also reduced, but to a lesser extent (Fig. 7A). To investigate whether the reduced amount

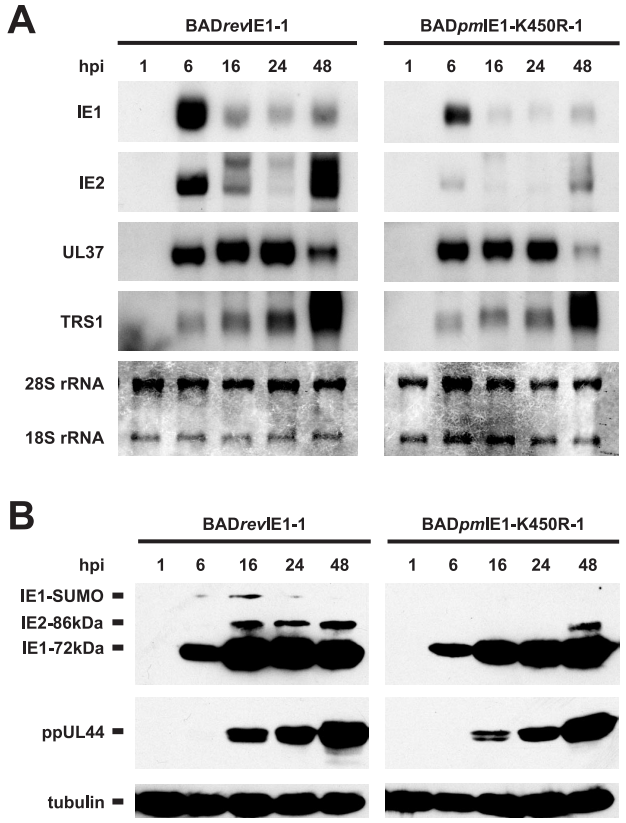

FIG. 7. Comparisons between the temporal pattern of viral RNA and protein accumulation in cells infected with the K450R mutant and a revertant virus. (A) Northern blots showing expression of HCMV IE1, IE2, UL37 exon 1, and TRS1 genes at the indicated times (hours) after infection (hpi) in fibroblasts infected with the revertant or K450R mutant virus (3 PFU/cell). As a loading control, 28S and 18S rRNAs were visualized by staining of the membranes with methylene blue solution (0.04% in 0.5 M sodium acetate, pH 5.2). (B) Western blot analysis showing expression of the HCMV IE1-72kDa (SUMOylated and nonconjugated), IE2-86kDa, and ppUL44 proteins by using antibodies MAB810 (for the IE1 and IE2 proteins) and anti-ICP36 (for ppUL44), respectively, at the indicated times after infection with wild-type and K450R mutant viruses (3 PFU/cell). A loading control (α-tubulin, DM1A) is also included.

of IE2 mRNA led to the accumulation of less protein, we monitored the accumulation of the major IE2 protein species, IE2-86kDa, by Western blotting in extracts of cells infected with BAD*pm*IE1-K450R and BAD*rev*IE1 (Fig. 7B). In accordance with the results from our RNA analyses, the accumulation of IE2-86kDa was markedly delayed and reduced in the mutant, while the levels of IE1-72kDa were, if at all, only slightly affected. In fact, there was no detectable IE2 protein up to 24 h after infection with BAD*pm*IE1-K405R at normal film exposures, compared to readily detectable levels at 16 h postinfection with the revertant virus. However, after 48 h the accumulation of IE2-86kDa approached wild-type levels, indicating that production of the IE2 protein was delayed but not completely blocked (Fig. 7B). Surprisingly, expression of the UL44 early viral protein was not significantly affected (Fig. 7B), despite the fact that IE2-86kDa has been unequivocally shown to be required for viral early gene expression (24, 38). This is most likely due to the fact that the small amounts of IE2 gene products that are detectable at the RNA (Fig. 7A) but not the protein (Fig. 7B) level are sufficient to transactivate viral early promoters. Interestingly, several independent experiments with different antibodies showed that the SUMOylated form of IE1-72kDa peaks in abundance at 10 to 18 h postinfection, a time frame that correlates with the initial accumulation of IE2-86kDa (Fig. 7B and data not shown). This observation provides further support for the view that SUMOylation of IE1-72kDa is linked to efficient expression of the IE2 gene product. In summary, the growth defect of the IE1-K450R mutant virus is, at least in part, due to delayed accumulation of IE2-86kDa.

DISCUSSION

It is well established that IE1-72kDa is required for normal progression of the HCMV lytic cycle (20, 22, 43), but the exact role of this protein in viral replication remains elusive. IE1-72kDa, like the other abundant HCMV major immediate-early gene product, IE2-86kDa, is a substrate for phosphorylation (46) and covalent conjugation to the SUMO-1 protein (5, 26, 44, 63, 73) (Fig. 2C and 7B). We have demonstrated that at least one aspect of its function requires SUMOylation.

Previously generated IE1-deficient viruses are null mutants that have been constructed by homologous recombination in mammalian cells followed by plaque purification (22, 43). In this report we have described the construction and characterization of the first IE1 mutant virus generated by homologous recombination in *E. coli* with a BAC system, and we have generated the first virus with point mutations in the IE1-specific coding sequence. Given the multiplicity-dependent phenotype of IE1 null mutant viruses (3, 20, 22, 43), it is not surprising that HCMV BACs lacking most of the IE1-coding region (pBAD*sub*IE1) failed to produce virus after transfection into noncomplementing permissive fibroblasts (Table 1). However, we were consistently able to reconstitute infectious virus from pBAD*pm*IE1-K450R, demonstrating that SUMO modification is not absolutely required for the infectivity of HCMV BAC DNA and productive viral replication (Table 1). However, SUMOylation of IE1-72kDa clearly contributes to the full activity of this protein at both high and low MOIs (Fig. 3). Therefore, it is conceivable that the SUMOylated and nonconjugated forms of IE1-72kDa may perform distinct roles during virus infection. The non-SUMOylated protein, which represents the predominant IE1 species in infected cells (Fig. 2C), may provide the main function that facilitates viral early gene expression and productive viral replication through SUMOylation-independent activities such as transcriptional activation of viral and cellular genes, ND10 disruption, and/or chromatin binding (20, 22, 43, 63, 73) (Fig. 4 and 5). In contrast, the low steady-state levels of SUMOylated IE1-72kDa evidently have an additional, supporting role that contributes to efficient lytic viral growth.

Our results indicate that this supporting function is linked to the induction of IE2 RNA accumulation (Fig. 7). This observation is somewhat surprising, since a previously characterized IE1 null virus (CR208) did not exhibit significantly reduced steady-state levels of IE2-86kDa (3, 20, 22). However, this recombinant virus was derived from the HCMV Towne strain, and an earlier mutant (RC303ΔAcc), which was constructed in

a Towne/Toledo hybrid virus background showed substantially reduced accumulation of IE2-86kDa that was attributed to a failure in autoregulation (43). Since BAD*pm*IE1-K450R is the first IE1 mutant virus that is based on HCMV strain AD169, it is conceivable that interstrain variations contribute to the differences in the observed phenotypes. In this context it is interesting that the IE2-86kDa proteins from different HCMV strains have recently been shown to differ remarkably in various biochemical and functional activities (8).

The effect of IE1 SUMOylation on IE2 expression is most likely a posttranscriptional event rather than a result of transcriptional activation, since both IE1 and IE2 are transcribed from the major immediate-early promoter and only IE2 expression is strongly reduced in the K450R mutant virus, while there is little or no effect on the IE1 RNA and protein levels (Fig. 7). Moreover, the K450R mutation did not adversely affect transactivation of the HCMV major immediate-early promoter by IE1-72kDa in our luciferase reporter assays (Fig. 6). Interestingly, it has been previously reported that normally discordant expression of the IE1 and IE2 genes at early times after infection involves posttranscriptional processing events (64). How SUMOylated IE1-72kDa exerts a posttranscriptional function is presently unknown. However, it is tempting to speculate that the SUMO moiety may mediate interactions with components of RNA-processing complexes just like, for example, SUMOylation of PML mediates the recruitment of other ND10-targeting proteins such as Sp100, Daxx, and CREB-binding protein (27, 78). Alternatively, it is conceivable that SUMOylated IE1-72kDa alters the expression of another viral or cellular gene product, which then influences the accumulation of IE2 mRNA and protein. Irrespective of its mode of action, our mutational analysis demonstrates that SUMOylation of IE1-72kDa facilitates viral replication by enhancing the expression of a second mRNA and protein, IE2-86kDa, derived from the same transcription unit.

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REFERENCES

1. **Adamson, A. L., and S. Kenney.** 2001. Epstein-Barr virus immediate-early protein BZLF1 is SUMO-1 modified and disrupts promyelocytic leukemia bodies. J. Virol. **75:**2388–2399.
2. **Ahn, J. H., E. J. Brignole III, and G. S. Hayward.** 1998. Disruption of PML subnuclear domains by the acidic IE1 protein of human cytomegalovirus is mediated through interaction with PML and may modulate a RING finger-dependent cryptic transactivator function of PML. Mol. Cell. Biol. **18:**4899–4913.
3. **Ahn, J. H., and G. S. Hayward.** 2000. Disruption of PML-associated nuclear bodies by IE1 correlates with efficient early stages of viral gene expression and DNA replication in human cytomegalovirus infection. Virology **274:**39–55.
4. **Ahn, J. H., and G. S. Hayward.** 1997. The major immediate-early proteins IE1 and IE2 of human cytomegalovirus colocalize with and disrupt PML-associated nuclear bodies at very early times in infected permissive cells. J. Virol. **71:**4599–4613.
5. **Ahn, J. H., Y. Xu, W. J. Jang, M. J. Matunis, and G. S. Hayward.** 2001. Evaluation of interactions of human cytomegalovirus immediate-early IE2 regulatory protein with small ubiquitin-like modifiers and their conjugation enzyme Ubc9. J. Virol. **75:**3859–3872.
6. **Atalay, R., A. Zimmermann, M. Wagner, E. Borst, C. Benz, M. Messerle, and H. Hengel.** 2002. Identification and expression of human cytomegalovirus transcription units coding for two distinct Fcγ receptor homologs. J. Virol. **76:**8596–8608.
7. **Baldick, C. J., Jr., A. Marchini, C. E. Patterson, and T. Shenk.** 1997. Human cytomegalovirus tegument protein pp71 (ppUL82) enhances the infectivity of viral DNA and accelerates the infectious cycle. J. Virol. **71:**4400–4408.
8. **Barrasa, M. I., N. Harel, Y. Yu, and J. C. Alwine.** 2003. Strain variations in single amino acids of the 86-kilodalton human cytomegalovirus major immediate-early protein (IE2) affect its functional and biochemical properties: implications of dynamic protein conformation. J. Virol. **77:**4760–4772.
9. **Bies, J., J. Markus, and L. Wolff.** 2002. Covalent attachment of the SUMO-1 protein to the negative regulatory domain of the c-Myb transcription factor modifies its stability and transactivation capacity. J. Biol. Chem. **277:**8999–9009.
10. **Bresnahan, W. A., I. Boldogh, E. A. Thompson, and T. Albrecht.** 1996. Human cytomegalovirus inhibits cellular DNA synthesis and arrests productively infected cells in late G1. Virology **224:**150–160.
11. **Bresnahan, W. A., and T. Shenk.** 2000. A subset of viral transcripts packaged within human cytomegalovirus particles. Science **288:**2373–2376.
12. **Brune, W., M. Messerle, and U. H. Koszinowski.** 2000. Forward with BACs: new tools for herpesvirus genomics. Trends Genet. **16:**254–259.
13. **Castillo, J. P., and T. F. Kowalik.** 2002. Human cytomegalovirus immediate early proteins and cell growth control. Gene **290:**19–34.
14. **Castillo, J. P., A. D. Yurochko, and T. F. Kowalik.** 2000. Role of human cytomegalovirus immediate-early proteins in cell growth control. J. Virol. **74:**8028–8037.
15. **Cherepanov, P. P., and W. Wackernagel.** 1995. Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene **158:**9–14.
16. **Desterro, J. M., M. S. Rodriguez, and R. T. Hay.** 1998. SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. Mol. Cell **2:**233–239.
17. **Duprez, E., A. J. Saurin, J. M. Desterro, V. Lallemand-Breitenbach, K. Howe, M. N. Boddy, E. Solomon, H. de The, R. T. Hay, and P. S. Freemont.** 1999. SUMO-1 modification of the acute promyelocytic leukaemia protein PML: implications for nuclear localisation. J. Cell Sci. **112:**381–393.
18. **Endter, C., J. Kzhyshkowska, R. Stauber, and T. Dobner.** 2001. SUMO-1 modification required for transformation by adenovirus type 5 early region 1B 55-kDa oncoprotein. Proc. Natl. Acad. Sci. USA **98:**11312–11317.
19. **Everett, R. D.** 2001. DNA viruses and viral proteins that interact with PML nuclear bodies. Oncogene **20:**7266–7273.
20. **Gawn, J. M., and R. F. Greaves.** 2002. Absence of IE1 p72 protein function during low-multiplicity infection by human cytomegalovirus results in a broad block to viral delayed-early gene expression. J. Virol. **76:**4441–4455.
21. **Gravel, A., J. Gosselin, and L. Flamand.** 2002. Human herpesvirus 6 immediate-early 1 protein is a sumoylated nuclear phosphoprotein colocalizing with promyelocytic leukemia protein-associated nuclear bodies. J. Biol. Chem. **277:**19679–19687.
22. **Greaves, R. F., and E. S. Mocarski.** 1998. Defective growth correlates with reduced accumulation of a viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus IE1 mutant. J. Virol. **72:**366–379.
23. **Hayhurst, G. P., L. A. Bryant, R. C. Caswell, S. M. Walker, and J. H. Sinclair.** 1995. CCAAT box-dependent activation of the TATA-less human DNA polymerase alpha promoter by the human cytomegalovirus 72-kilodalton major immediate-early protein. J. Virol. **69:**182–188.
24. **Heider, J. A., W. A. Bresnahan, and T. E. Shenk.** 2002. Construction of a rationally designed human cytomegalovirus variant encoding a temperature-sensitive immediate-early 2 protein. Proc. Natl. Acad. Sci. USA **99:**3141–3146.
25. **Hobom, U., W. Brune, M. Messerle, G. Hahn, and U. H. Koszinowski.** 2000. Fast screening procedures for random transposon libraries of cloned herpesvirus genomes: mutational analysis of human cytomegalovirus envelope glycoprotein genes. J. Virol. **74:**7720–7729.
26. **Hofmann, H., S. Floss, and T. Stamminger.** 2000. Covalent modification of the transactivator protein IE2-p86 of human cytomegalovirus by conjugation to the ubiquitin-homologous proteins SUMO-1 and hSMT3b. J. Virol. **74:**2510–2524.
27. **Ishov, A. M., A. G. Sotnikov, D. Negorev, O. V. Vladimirova, N. Neff, T. Kamitani, E. T. Yeh, J. F. Strauss III, and G. G. Maul.** 1999. PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. J. Cell Biol. **147:**221–234.
28. **Kelly, C., R. Van Driel, and G. W. Wilkinson.** 1995. Disruption of PML-associated nuclear bodies during human cytomegalovirus infection. J. Gen. Virol. **76:**2887–2893.
29. **Kim, K. I., S. H. Baek, and C. H. Chung.** 2002. Versatile protein tag, SUMO: its enzymology and biological function. J. Cell Physiol. **191:**257–268.
30. **Kondo, K., and E. S. Mocarski.** 1995. Cytomegalovirus latency and latency-

specific transcription in hematopoietic progenitors. Scand. J. Infect. Dis. Suppl. **99:**63–67.
31. **Korioth, F., G. G. Maul, B. Plachter, T. Stamminger, and J. Frey.** 1996. The nuclear domain 10 (ND10) is disrupted by the human cytomegalovirus gene product IE1. Exp. Cell Res. **229:**155–158.
32. **Lafemina, R. L., M. C. Pizzorno, J. D. Mosca, and G. S. Hayward.** 1989. Expression of the acidic nuclear immediate-early protein (IE1) of human cytomegalovirus in stable cell lines and its preferential association with metaphase chromosomes. Virology **172:**584–600.
33. **Lallemand-Breitenbach, V., J. Zhu, F. Puvion, M. Koken, N. Honore, A. Doubeikovsky, E. Duprez, P. P. Pandolfi, E. Puvion, P. Freemont, and H. de The.** 2001. Role of promyelocytic leukemia (PML) sumolation in nuclear body formation, 11S proteasome recruitment, and As2O3-induced PML or PML/retinoic acid receptor alpha degradation. J. Exp. Med. **193:**1361–1371.
34. **Lee, E. C., D. Yu, J. Martinez de Velasco, L. Tessarollo, D. A. Swing, D. L. Court, N. A. Jenkins, and N. G. Copeland.** 2001. A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics **73:**56–65.
35. **Lu, M., and T. Shenk.** 1996. Human cytomegalovirus infection inhibits cell cycle progression at multiple points, including the transition from G_1 to S. J. Virol. **70:**8850–8857.
36. **Lu, M., and T. Shenk.** 1999. Human cytomegalovirus UL69 protein induces cells to accumulate in G_1 phase of the cell cycle. J. Virol. **73:**676–683.
37. **Lukac, D. M., N. Y. Harel, N. Tanese, and J. C. Alwine.** 1997. TAF-like functions of human cytomegalovirus immediate-early proteins. J. Virol. **71:** 7227–7239.
38. **Marchini, A., H. Liu, and H. Zhu.** 2001. Human cytomegalovirus with IE-2 (UL122) deleted fails to express early lytic genes. J. Virol. **75:**1870–1878.
39. **Margolis, M. J., S. Pajovic, E. L. Wong, M. Wade, R. Jupp, J. A. Nelson, and J. C. Azizkhan.** 1995. Interaction of the 72-kilodalton human cytomegalovirus IE1 gene product with E2F1 coincides with E2F-dependent activation of dihydrofolate reductase transcription. J. Virol. **69:**7759–7767.
40. **Messerle, M., G. Hahn, W. Brune, and U. H. Koszinowski.** 2000. Cytomegalovirus bacterial artificial chromosomes: a new herpesvirus vector approach. Adv. Virus Res. **55:**463–478.
41. **Mitsudomi, T., S. M. Steinberg, M. M. Nau, D. Carbone, D. D'Amico, S. Bodner, H. K. Oie, R. I. Linnoila, J. L. Mulshine, J. D. Minna, et al.** 1992. p53 gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of ras mutations and clinical features. Oncogene **7:**171–180.
42. **Mocarski, E. S., and C. T. Courcelle.** 2001. Cytomegaloviruses and their replication, p. 2629–2673. *In* D. M. Knipe, P. M. Howley, et al. (ed.), Fields virology, 4th ed., vol. 2. Lippincott Williams and Wilkins, Philadelphia, Pa.
43. **Mocarski, E. S., G. W. Kemble, J. M. Lyle, and R. F. Greaves.** 1996. A deletion mutant in the human cytomegalovirus gene encoding IE1(491aa) is replication defective due to a failure in autoregulation. Proc. Natl. Acad. Sci. USA **93:**11321–11326.
44. **Muller, S., and A. Dejean.** 1999. Viral immediate-early proteins abrogate the modification by SUMO-1 of PML and Sp100 proteins, correlating with nuclear body disruption. J. Virol. **73:**5137–5143.
45. **Muller, S., M. J. Matunis, and A. Dejean.** 1998. Conjugation with the ubiquitin-related modifier SUMO-1 regulates the partitioning of PML within the nucleus. EMBO J. **17:**61–70.
46. **Pajovic, S., E. L. Wong, A. R. Black, and J. C. Azizkhan.** 1997. Identification of a viral kinase that phosphorylates specific E2Fs and pocket proteins. Mol. Cell. Biol. **17:**6459–6464.
47. **Pari, G. S., and D. G. Anders.** 1993. Eleven loci encoding *trans*-acting factors are required for transient complementation of human cytomegalovirus ori-Lyt-dependent DNA replication. J. Virol. **67:**6979–6988.
48. **Pichler, A., and F. Melchior.** 2002. Ubiquitin-related modifier SUMO1 and nucleocytoplasmic transport. Traffic **3:**381–387.
49. **Poma, E. E., T. F. Kowalik, L. Zhu, J. H. Sinclair, and E. S. Huang.** 1996. The human cytomegalovirus IE1–72 protein interacts with the cellular p107 protein and relieves p107-mediated transcriptional repression of an E2F-responsive promoter. J. Virol. **70:**7867–7877.
50. **Posfai, G., M. D. Koob, H. A. Kirkpatrick, and F. R. Blattner.** 1997. Versatile insertion plasmids for targeted genome manipulations in bacteria: isolation, deletion, and rescue of the pathogenicity island LEE of the *Escherichia coli* O157:H7 genome. J. Bacteriol. **179:**4426–4428.
51. **Rangasamy, D., and V. G. Wilson.** 2000. Bovine papillomavirus E1 protein is sumoylated by the host cell Ubc9 protein. J. Biol. Chem. **275:**30487–30495.
52. **Rangasamy, D., K. Woytek, S. A. Khan, and V. G. Wilson.** 2000. SUMO-1 modification of bovine papillomavirus E1 protein is required for intranuclear accumulation. J. Biol. Chem. **275:**37999–38004.
53. **Reddehase, M. J.** 2000. The immunogenicity of human and murine cytomegaloviruses. Curr. Opin. Immunol. **12:**390–396.
54. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27:**493–497.
55. **Regad, T., and M. K. Chelbi-Alix.** 2001. Role and fate of PML nuclear bodies in response to interferon and viral infections. Oncogene **20:**7274–7286.
56. **Rogan, S., and S. Heaphy.** 2000. The vaccinia virus E3L protein interacts with SUMO-1 and ribosomal protein L23a in a yeast two hybrid assay. Virus Genes **21:**193–195.
57. **Romanowski, M. J., and T. Shenk.** 1997. Characterization of the human cytomegalovirus irs1 and trs1 genes: a second immediate-early transcription unit within irs1 whose product antagonizes transcriptional activation. J. Virol. **71:**1485–1496.
58. **Sarisky, R. T., and G. S. Hayward.** 1996. Evidence that the UL84 gene product of human cytomegalovirus is essential for promoting oriLyt-dependent DNA replication and formation of replication compartments in cotransfection assays. J. Virol. **70:**7398–7413.
59. **Schwartz, D. C., and M. Hochstrasser.** 2003. A superfamily of protein tags: ubiquitin, SUMO and related modifiers. Trends Biochem. Sci. **28:**321–328.
60. **Seeler, J. S., and A. Dejean.** 2003. Nuclear and unclear functions of SUMO. Nat. Rev. Mol. Cell. Biol. **4:**690–699.
61. **Shen, Y., H. Zhu, and T. Shenk.** 1997. Human cytomegalovirus IE1 and IE2 proteins are mutagenic and mediate "hit-and-run" oncogenic transformation in cooperation with the adenovirus E1A proteins. Proc. Natl. Acad. Sci. USA **94:**3341–3345.
62. **Shirakata, M., M. Terauchi, M. Ablikim, K. Imadome, K. Hirai, T. Aso, and Y. Yamanashi.** 2002. Novel immediate-early protein IE19 of human cytomegalovirus activates the origin recognition complex I promoter in a cooperative manner with IE72. J. Virol. **76:**3158–3167.
63. **Spengler, M. L., K. Kurapatwinski, A. R. Black, and J. Azizkhan-Clifford.** 2002. SUMO-1 modification of human cytomegalovirus IE1/IE72. J. Virol. **76:**2990–2996.
64. **Stamminger, T., E. Puchtler, and B. Fleckenstein.** 1991. Discordant expression of the immediate-early 1 and 2 gene regions of human cytomegalovirus at early times after infection involves posttranscriptional processing events. J. Virol. **65:**2273–2282.
65. **Stuurman, N., A. de Graaf, A. Floore, A. Josso, B. Humbel, L. de Jong, and R. van Driel.** 1992. A monoclonal antibody recognizing nuclear matrix-associated nuclear bodies. J. Cell Sci. **101:**773–784.
66. **Szostecki, C., H. H. Guldner, H. J. Netter, and H. Will.** 1990. Isolation and characterization of cDNA encoding a human nuclear antigen predominantly recognized by autoantibodies from patients with primary biliary cirrhosis. J. Immunol. **145:**4338–4347.
67. **Verger, A., J. Perdomo, and M. Crossley.** 2003. Modification with SUMO. A role in transcriptional regulation. EMBO Rep. **4:**137–142.
68. **Wagner, M., Ruzsics, Z., Koszinowski, U. H.** 2002. Herpesvirus genetics has come of age. Trends Microbiol. **10:**318–324.
69. **Wiebusch, L., and C. Hagemeier.** 1999. Human cytomegalovirus 86-kilodalton IE2 protein blocks cell cycle progression in G_1. J. Virol. **73:**9274–9283.
70. **Wilkinson, G. W., C. Kelly, J. H. Sinclair, and C. Rickards.** 1998. Disruption of PML-associated nuclear bodies mediated by the human cytomegalovirus major immediate early gene product. J. Gen. Virol. **79:**1233–1245.
71. **Wilson, V. G., and D. Rangasamy.** 2001. Intracellular targeting of proteins by sumoylation. Exp. Cell Res. **271:**57–65.
72. **Wilson, V. G., and D. Rangasamy.** 2001. Viral interaction with the host cell sumoylation system. Virus Res. **81:**17–27.
73. **Xu, Y., J. H. Ahn, M. Cheng, C. M. apRhys, C. J. Chiou, J. Zong, M. J. Matunis, and G. S. Hayward.** 2001. Proteasome-independent disruption of PML oncogenic domains (PODs), but not covalent modification by SUMO-1, is required for human cytomegalovirus immediate-early protein IE1 to inhibit PML-mediated transcriptional repression. J. Virol. **75:**10683–10695.
74. **Yu, D., H. M. Ellis, E. C. Lee, N. A. Jenkins, N. G. Copeland, and D. L. Court.** 2000. An efficient recombination system for chromosome engineering in Escherichia coli. Proc. Natl. Acad. Sci. USA **97:**5978–5983.
75. **Yu, Y., and J. C. Alwine.** 2002. Human cytomegalovirus major immediate-early proteins and simian virus 40 large T antigen can inhibit apoptosis through activation of the phosphatidylinositide 3′-OH kinase pathway and the cellular kinase Akt. J. Virol. **76:**3731–3738.
76. **Yurochko, A. D., M. W. Mayo, E. E. Poma, A. S. Baldwin, Jr., and E. S. Huang.** 1997. Induction of the transcription factor Sp1 during human cytomegalovirus infection mediates upregulation of the p65 and p105/p50 NF-κB promoters. J. Virol. **71:**4638–4648.
77. **Zhang, Y., F. Buchholz, J. P. Muyrers, and A. F. Stewart.** 1998. A new logic for DNA engineering using recombination in Escherichia coli. Nat. Genet. **20:**123–128.
78. **Zhong, S., S. Muller, S. Ronchetti, P. S. Freemont, A. Dejean, and P. P. Pandolfi.** 2000. Role of SUMO-1-modified PML in nuclear body formation. Blood **95:**2748–2752.
79. **Zhu, H., Y. Shen, and T. Shenk.** 1995. Human cytomegalovirus IE1 and IE2 proteins block apoptosis. J. Virol. **69:**7960–7970.